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THE PHYSIOLOGICAL AND SYMBIOTIC RELATIONSHIPS BETWEEN THE INTESTINAL PROTOZOA OF TERMITES AND THEIR HOST, WITH SPECIAL REFERENCE TO RETICULITERMES FLAVIPES KOLLAR

DISSERTATION

Submitted to the Director of the School of Hygiene
and Public Health of the Johns Hopkins University
in Conformity with the Requirements for the Degree
of Doctor of Science in Hygiene.

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June 1923.

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INTRODUCTION

The most unusual and peculiarly interesting faunal association known to parasitology is that represented by the teeming menagerie of intestinal protozoa harbored by termites. These protozoa are favorite objects for study and have attracted the attention of many investigators. The initial microscopic view of thousands of these organisms, but recently removed from the termite's intestine, wriggling, undulating, and twisting in a drop of saline solution, never fails to fill the observer with enthusiasm, and bring forth volleys of questions. Perhaps the first thing to attract attention is the abundance, both in form and number, of protozoa present in a single host. The large abdomen is almost completely filled by the greatly distended intestine, the lumen of which is gorged by a vast horde of protozoa.

The protozoa of the termites, for the most part, differ considerably from those found in other animals, and this difference cannot fail to attract the attention of a protozoologist. Their most distinctive features are: the ingestion of solid particles of wood for food; the diversity of organization among the various forms; the degree of structural

specialization* attained by many of the species, resulting in the formation of the most complex flagellate organelles yet known.

No observer has ever failed to raise the question of the relation of the protozoa to their host, but most investigators in the past have regarded the protozoa either as true parasites or commensals, though, in almost every instance, no proof whatever is offered in support of either contention.

At present no termite of the most highly specialized family, the Termitidae (Metatermitidae Holmgren), is known to harbor intestinal protozoa; but all the genera and species of the other families, Kalotermitidae (Protermitidae Holmgren), Rhinotermitidae (Mesotermitidae Holmgren) and Mastotermitidae, that have been examined# have been found to harbor enormous numbers of protozoa. It is not definitely known why protozoa are never found in the intestine of any of the Termitidae; but a difference in the feeding habit has been suggested as a very probable explanation, since few, if any, of the Termitidae feed solely on wood.

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* See especially the papers of Kofoid and Swezy (1919) describing the neuromotor system of Trichonympha campanula and Trichomitus termitidis.

See the first paper of this series, "Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa", for a list of the genera and species that have been examined. Since writing the above more than 75 species of the Termitidae have been examined and 1 out of 17 species of Nasutitermes and 2 out of 7 species of Mirotermes have been found to feed on wood and harbor protozoa.

THE PROBLEM

To determine the relation of the intestinal protozoa of termites to their host.

Experimental methods were the paramount mode of attack; and it was necessary, in order to solve the large problem stated above, to solve several problems, which may be briefly stated as follows:

1. What is the principal compound in wood, the sole article of diet of most termites, which is used for food?

2. How do termites utilize cellulose, the principal compound in wood, as their chief article of food?

3. How may all of the intestinal protozoa be killed without at the same time injuring their host? To develop a rapid and successful method for removing the protozoa from the termites was a very difficult proposition.

4. Why do termites, when subjected to a temperature of 36 degrees C. for 24 hours, lose their ability to maintain themselves on a wood diet?

5. Is defaunation (the removal of the protozoan fauna), which occurs when termites are incubated at 36 degrees C. for 24

hours, in any way responsible for the inability of the termites to maintain themselves on a wood diet?

6. Why do the incubated and defaunated termites live indefinitely when fed the decomposition products of wood, or the products of fungus-digested cellulose; but die within 10-20 days when fed wood, their normal diet, or when fed cellulose?

7. Why do the incubated and defaunated termites regain their ability to make use of wood or pure cellulose as food when reinfected (refaunated) with protozoa?

8. Do the unincubated and faunated termites harbor any intestinal bacteria or fungi, capable of digesting cellulose, which are killed off during incubation?

9. Do the protozoa aid their host mechanically to digest wood, or do the protozoa digest the wood particles which they take into their bodies and convert them into substances which their host uses for food?

10. Since the termites lose their ability to digest wood when the protozoa are removed from their intestine, that is when they are defaunated, but regain this ability when reinfected with protozoa, and since the protozoa themselves can be shown to

digest the wood particles which they take into their bodies, does a true symbiotic relationship exist between termites and their intestinal protozoa?

MATERIAL

Reticulitermes flavipes Kollar, collected in Maryland, near Baltimore, was used for all the experimental work on this genus, though many colonies from other states have been used for studying the protozoa of this species. *Termopsis sp.* (the species could not be determined because there were no winged adults in any of the colonies collected, but it is either *angusticollis* Walker or *nevadensis* Hagen) from Ashland, Oregon, owing to its large size, was used in many of the feeding experiments, though *Reticulitermes flavipes* was also used in all of the feeding experiments. *Kalotermes schwarzi* Banks, *K. jouteli* Banks and *Prorhinotermes simplex* Hagen, from southern Florida, were used for incubation experiments, but the material of these genera was not sufficient to make possible as extensive a study as was carried out with the material from Maryland. *Amitermes* sp., from Uvalde, Texas, and *Nasutitermes morio* Latreille, from Porto Rico, both of the family Termitidae, were studied.

ACKNOWLEDGMENTS

Many thanks are due Drs. R. W. Hegner and W. H. Taliaferro, of the Department of Medical Zoology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland, for valuable suggestions and criticisms. I am also very deeply indebted to Dr. T. E. Snyder, Specialist in Forest Insects, Bureau of Entomology, U. S. Department of Agriculture, Washington, D. C., who identified all the termites studied and furnished all the material except *Reticulitermes flavipes* and *Nasutitermes morio*.

GENERAL CONSIDERATIONS

1. The Termites

a. Castes

In order that the reader, unfamiliar with the nature of a termite colony, may know something of the castes present, the following condensed statement (taken from the papers of Snyder and Thompson) is made. In *Reticulitermes flavipes* there are five castes, three of which are fertile or reproductive, and two that are sterile or non-reproductive. Males and females occur in each of the five castes. The reproductive castes are: (1) the *first form*, which has three well defined phases of development; (a) the nymphs, with long wing pads, creamy white

body 1.3-1.4 mm. long, and light brown eyes; (b) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (c) the older males and females, with enlarged abdomens and the scales of the shed wings, body 7-14 mm. long; (2) the *second form*, which, like the first form, has three well defined phases of development: (a) the nymphs, with short wing pads and colorless body and eyes; (b) the young adults, with short scaly wing vestiges, straw colored or grayish body 6-7 mm. long; (c) the older adults, with wing vestiges, enlarged abdomen, body length 7-12 mm: (3) the *third form*, which also has three developmental phases: (a) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (b) the young adults, wingless, head and body pure white, opaque and not transparent, about 6 mm. long; (c) the older adults, with enlarged abdomen, wingless, head and body white, 7-9 mm. long. The other castes, tho sterile castes, are: (4) the *worker*, wingless with grayish abdomen, only two developmental phases, i.e., nymphs and adults, always feeds on wood and there is no degeneration of the jaw muscles, always harbors protozoa once an infestation has been acquired, salivary glands small and very little fatty tissue is present in the body; (5) *soldier*, wingless with elongated head covered with thick yellowish chitin, mandibles dark brown long slender and curved,

abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth.

Thompson (1917) showed that the newly hatched nymphs of Reticulitermes flavipes, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (a) reproductive nymphs, from which develop the three fertile adult castes, and (b) the worker-soldier nymphs, from which develop the two sterile adult castes. By the time the reproductive nymphs had attained a body length of 1.3-1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated, internally, when they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

Several intermediate forms have been discovered by various investigators, intermediates between the first and second forms, three fertile soldiers (Heath 1902), and others; but these need not be considered here.

The five castes occur in most termites. Known exceptions are as follows: the third form reproductive caste

occurs in few, if any, of the Termitidae; in fact none have been reported from the Nearctic genera of this family, and it is doubtful if this caste occurs in any genus of the Termitidae. Apparently this caste has been lost in this, the most highly specialized, family of termites. The third form is less common than the first and second in *Reticulitermes flavipes*, but in *Termopsis* it is more common than the first and second form. *Reticulitermes* is about midway between the most primitive and the most highly specialized termites. *Termopsis* is a primitive genus. A true worker caste is not present in *Termopsis* and *Neotermes*, but a large-headed worker-like reproductive form is present. Two genera, *Kalotermes* and *Cryptotermes*, have no worker caste. The genus *Anoplotermes* has no soldier caste.

Grassi calls the first forms "true" or "perfect insects", or "the royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms he thinks are always ready to take the place of the royal forms in case of need. He recognizes nymphs of the first and second forms, but these, he thinks, if taken young enough and given the proper food, may be changed into anything else, i.e., any of the castes. But, Thompson (1919 and 1922) and Snyder (1920) have presented valuable experimental evidence in support of the view that each of the

three reproductive castes breeds true, that is, produces reproductive adults only like itself.

b. Food - direct

Wood is a direct food of most termites. Some of them feed on dry heart wood, while others feed on moist and more decayed wood, usually sap wood. *Kalotermes schwarzi*, for instance, is often found in furniture stored in an attic, since it does not require access to the soil in nature as does *Reticulitermes flavipes*. However it is quite probable that *R. flavipes* requires access to the soil only in order to obtain moisture, since it may be kept in captivity indefinitely without access to soil provided sufficient moisture is given it. Water, of course, is a direct food, though some termites require very little of it. *Amitermes tubiformans* Buckley, for example, feeds principally on dry cow chips and soil, but occasionally eats the roots of grass and other vegetation. The manure is often carried down into chambers below the ground. Many of the Termitidae feed on plants, sometimes attacking flowers, shrubs and field crops. Fungus gardens are cultivated by many species of this family. These termites carry large quantities of vegetation into their huge nests and

take advantage of the digestive action of the fungus on the hemicellulose, the fungus and hemicellulose being eaten by them as food. It is interesting to note that leaf-cutting ants (Oecodoma) do the same thing. They carry thousands of leaves to their nest, where they use them to make a compost heap, on which fungi, to which the ants are very partial, grow. Termites in captivity may be induced to eat almost anything, but our knowledge regarding their habits in nature, especially the Termitidae, is so limited that it is impossible to adequately discuss their food.

c. Food - indirect

The liquid diet is either a salivary secretion or regurgitated material from the mid-gut. It is very probably the former. It is a colorless and distinctly alkaline liquid which collects on the labrum as a small drop, which may be used either as food for others or for the termite secreting it. Many termites also employ this liquid in building. In Reticulitermes flavipes the young larvae of all the castes and the second and third forms during the third developmental stage feed solely on this liquid diet which they receive from other members of the colony. Later, the larvae of all the castes eat the excreta as it is passed from the anus of the

older members of the colony, and it is at this time and in this way that they first become infected with protozoa. The larvae also begin to eat wood at or about the same time that they begin to eat excrement - which in this termite is composed of a few wood particles, but primarily of protozoa and liquid. The soldiers, workers and first form reproductive males and females always feed on wood, though they - expecially the first forms during the third developmental stage - partake of a liquid diet as well. The exuviae are usually eaten and, sometimes, the dead bodies of other members of the colony are eaten.

2. The Protozoa

Lespes (1856) made the first recorded observation of termite protozoa, though he did not describe them. Leidy (1877 and 1881) was the first in America and the second in the world to observe these organisms. In 1881 he gave descriptions of the protozoa which he had observed in Reticulitermes flavipes, creating three genera for them, Trichonympha, Pyrsonympha and Dinenympha. This termite really harbors several other genera, most of which were included in Leidy's drawings as stages in the life cycle of the three genera mentioned above. More than a score of other investigators have studied these protozoa, and to date 41 genera have been de-

scribed. For a complete account of the work done by all the students of termite protozoa and for a classification of all of the protozoa which have been described from termites the reader is referred to the first article of this series, "The Correlation between the Food and Morphology of Termites and the Presence of Intestinal Protozoa."

All of the known termite protozoa, except *Gregarina termitis* and *Nyctotherus termitis*, have been placed in two orders of flagellates, the *Polymastigina* and the *Hypermastigina*, the latter being the most highly specialized order of flagellate protozoa.

BIOCHEMICAL CONSIDERATIONS

Dore (1920) gives the following analysis of yellow pine (*Pinus ponderosa*) oven-dry (100° C.) basis. Results in percentages.

Benzene extract ---------------2.22
Alcohol extract ---------------1.49
Cellulose -------------------57.72
Lignin ----------------------29.47
Xylan ------------------------3.49
Mannan -----------------------6.37
Galactan ---------------------0.78

The two following examples are taken from Ritter and Fleck (1922), who have analyzed a large number of American woods:

Analysis of a mean of four samples. Results expressed in percentages of oven-dry (105° C.) material.

	western yellow pine (*Pinus ponderosa*)	tanbark oak (*Quercus densiflora*)
Moisture	6.42	3.66
Ash	0.46	0.83
Solubility in cold water	4.09	4.10
Solubility in hot water	5.05	5.60
Solubility in ether	8.52	0.80
Solubility in 1% NaOH	20.30	23.96
Acetic acid	1.09	5.23
Methoxy	4.49	5.74
Pentosan	7.35	19.59
Methyl pentosan	1.62	none
Lignin	26.65	24.85
Cellulose	57.41	58.03

In the cellulose:

Pentosan	6.82	22.82
Methyl pentosan	1.98	none
Alpha-cellulose	62.10	56.77
Beta-cellulose	10.56	19.92
Gamma-cellulose	30.13	23.93

That the principal food of most termites is cellulose is a fact which appears to have escaped the attention of physiologists and biochemists. Because of this, and also owing to the fact that our text-books of chemistry and physiology give little, if any, of the information which has been gained in recent researches on the chemistry and digestion of cellulose, it seems expedient to briefly mention - with no pretention at a discussion - some of the salient features brought out by a few of the more important of these researches.

According to Hibbert (1921) "cellulose is nothing more than a polmerized dextrose glucoside of dextrose," and Irvin and Hirst (1922) in their most recent paper on the constitution of polysaccharides have shown this to be true. They state that "the average yield from the polysaccharide (e.g., cellulose) to the hexose (e.g., glucose) is thus 95.5% of the theoretical amount. Considering the standard of purity in which the mixed methylglucosides were isolated, there can be no further doubt that cotton cellulose is composed entirely of glucose residues."

Pringsheim (1912) observed cellobiose - a disaccharide which bears the same relation to cellulose that maltose does to starch and which splits into two molecules of glucose (Fischer and Zemplen, 1904 and 1910), when acted upon by cellobiase - as an intermediate product of bacterial cellulose digestion. The exact mode of linking together of the two glucose residues in cellobiose was determined by Haworth and Hirst (1921). Groenewege (1920) showed that the bacterial digestion of cellulose is a hydrolytic process.

Cellulase is known to be produced by various moulds, soil fungi, pathogenic fungi, actinomyctes, soil and intestinal bacteria. Cellase is to be distinguished from cellulase in that it acts on

the hemicelluloses and is not an endo-enzyme (Pringsheim, 1912).

Some rather divergent views have been expressed regarding the products of cellulose digestion; different investigators working with different cellulose-decomposing organisms have gotten different results. But according to Cross and Doree (1922) most of these differences may be accounted for. The typical result of the digestion of wood cellulose is about as follows: Cellobiose, glucose, acetic acid, lactic acid, butyric acid, alcohol, CO_2, H_2, CH_4, CO. Under excessive aeration alcohol and acetic acid are completely oxidized to CO_2 and H_2O. The gas liberated is never pure CO_2; there is either H_2 or CH_4, or a mixture of the two. Alcohol is never obtained without acetic acid. Waksman (1922) is of the opinion that some soil bacteria cause CH_4 fermentation of cellulose and that others cause H_2 fermentation. McBeth (1913) thinks cellulose-fermenting organisms are not responsible for the production of gases. Gas, he thinks, is produced by contaminating organisms.

Abstracting Klason's remarkable researches on lignin, Cross and Doree (1922) state: "Beta-lignin ($C_{19}H_{18}O_9$, and that part of lignin precipitated by arylamine bases is designated

as acrolein-lignin or alpha-lignin. The other part, which is not precipitated, appears to be a carboxyl group and is called carboxyl lignin or beta-lignin) must therefore be bound to cellulose and probably to alpha-lignin ($C_{22}H_{22}O_7$). Lignin cannot well be assumed as a secondary product derived from cellulose, but appears as a direct assimilation product of CO_2 and H_2 or formaldehyde. Therefore the formation of lignin is a function of chlorophyll." Klason thinks lignin is possibly present in wood as a glucoside and that it may be built up from pentose.

THE RELATION OF THE PROTOZOA TO THEIR HOST

A. HISTORICAL

1. True Parasites.

Most students of termites, and termite protozoa, have focused their attention on the morphology* and behavior of either the termites or the protozoa which they harbor; consequently, the interrelation of host and parasite has been very little investigated.

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* The morphology of termite protozoa is a fascinating subject and occupied the writer's entire attention for a year before the present investigation began. He will publish in a later paper a description of several new forms.

Grassi is really the only investigator who has not confined his attention almost exclusively to a study of either the termites or their intestinal protozoa. He has studied the termites and the protozoa, but has never carefully investigated their relationship. His work on the two groups of organisms, termites and termite protozoa, has been largely a study of two separate and distinct problems; namely, the morphology and systematics of the protozoa, and the physiology and behavior of the termites, with special emphasis on the underlying factors in caste production.

Grassi and Sandias (1893) think that the relative abundance of protozoa present in the various adult individuals in a termite colony is inversely proportional to the degree of gonad development of their host. According to these investigators, the protozoa, with a small amount of saliva, function in the development of soldiers and workers. Why the same thing (many protozoa and a small amount of saliva) functions in the production of two separate and distinct castes, they do not consider. They observed that the protozoa were scarce in the winged sexual forms, and usually

totally absent in the neoteinic* individuals. When they found protozoa abundant in the neoteinic individuals, the gonad development was imperfect. They remarked that the developing neoteinic forms were fed largely on a salivary diet, which they thought killed the protozoa, though it is more probable that the protozoa died because their host no longer furnished them with wood for food. Then, according to Grassi and Sandias, it is impossible to say whether the maturation of the gonads is due solely to the change from a wood to a salivary diet or to the absence of protozoa. In this paper they reach no definite conclusion, though they do not believe the presence or absence of protozoa a sufficient cause to control gonad development, for they say, "It is a moot point whether the maturation of the generative organs is due solely to the saliva or to the absence of protozoa as well; but the latter, as my@ previous remarks show, is not by itself a sufficient cause." Then in the next paragraph, "I have fre-

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* The term neoteinia was first introduced by Camerano (Bull. Soc. Ent. Ital., 1885, pp. 89-94) to note the persistence during adult life of part or all of the characteristics normally peculiar to the immature, growing, or larval stages. Grassi and Sandias probably refer to the second and third form reproductive castes since it has been shown by Thompson (1920) that the jaw muscles of these forms degenerate to the extent that they cannot eat wood and must be fed on a fluid diet; and, of course, we should not expect them to harbor intestinal protozoa when they do not eat wood.

@ Grassi wrote most of the paper. The part written by Sandias is placed between asterisks.

quently asked myself whether the protozoa have not an important digestive function, since the comminuted wood passes almost entirely through their bodies. It is probable, but not proved."

Brunelli (1905) followed up the idea of Grassi and Sandias, and states that in the queens of two genera harboring protozoa, *Kalotermes flavicollis* and *Reticulitermes lucifugus*, there is a correlated destruction of the oocytes - a kind of indirect parasitic castration ("castration parasitaire"). Feutand (1912) says what Brunelli regards as signs of degeneration is in reality nothing more than an alteration of tissue brought about by the histological reagents.

Grassi, nineteen years later (1911), collaborating with Foa, is of the opinion that the protozoa do not aid their host in the digestion of food, and he points out that many wood feeding insects do not harbor protozoa. He states* that several workers (species not mentioned) of *Kalotermes* # were placed in small boxes with wood and incubated at about (*circa*) 35° C. It is interesting to note that none of the protozoa harbored by

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* Per mio conto in questi ultimi anni ho cercato di decidere la questione in altro modo: se si tengono ad una temperatura di circa 35° C. scatolette contenenti legno pieno di *Calotermes*, i Protozoi muoiono, talvolta tutti, più spesso restano in vita soltanto le forme piccole; si hanno così delle colonie di *Calotermes* senza *Joenia* e *Mesojoenia*, e talvolta con molti individui anche del tutto privi di Protozoi. Io le tengo in vita prospera da parecchi mesi; perciò ritengo che i *Calotermes* possano digerire il legno anche senza gli speciali Protozoi (*Joenia* e *Mesojoenia*).

Also spelled *Calotermes*.

Kalotermes schwarzi Banks, from Miami Beach, Florida, are killed when incubated for three days at 35 degrees C. How long Grassi's species of Kalotermes was incubated is not known, for he does not mention this in his paper. But he does state that the incubation killed off nearly all of the protozoa, all of the large forms being killed off every time. These termites, he states, lived in full activity for several months.

Recently Jucci (1920 and 1921), in a preliminary paper reported before the Academy of Lincei, has announced the discovery of a particular diet which brings about caste production. But the carefully controlled breeding experiments and the numerous field observations of Thompson and Snyder (1919, 1920, etc.) make it highly probable that at least three of the castes, namely, the first, the second and the third form reproductive males and females, only give rise to fertile reproductive males and females like themselves and to infertile males and females of the soldier and worker castes.

2. Commensals

If these entozoic protozoa benefit from their association with the termites, and by so doing do not harm or benefit their host, they are commensals. They may feed from

the waste food-products and bacteria in the termite's intestine, or they may be food-robbers and feed from the same table as their host; in either case, they are commensals, provided the host's table always contains sufficient food for both host and parasite. Kofoid and Swezy (1919) are the principal exponents of the commensalistic relationship of termite protozoa. They remark, "One of the most curious and unique faunal associations to be found among the parasitic* Protozoa is the group parasitic* or commensal in the intestinal tract of the social termites.... This distension is caused by the vast numbers of parasitic and commensal protozoans which fill the lumen of the intestine. When this is opened a thick milky fluid exudes. Under the lens this is found to be composed of great quantities of these small forms, thickly massed together, along with fragments of wood upon which the host, as well as some of the commensals, feeds."

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* This term, loosely used, means any organism which lives in or on another organism.

3. Symbionts.

Imms (1919) in studying the termite Archotermopsis wroughtoni Desneux made sections of twelve worker-like females and found numerous protozoa and nearly full sized ova ready for fertilization, and in no instance did he observe even an indication of oocyte degeneration. He also found well developed ovaries in five soldiers, whose hind-intestines were filled with protozoa. Imms suggests that the presence of protozoa may be correlated with the wood feeding habit of the termites. In the young larvae, and in the queens and kings, which receive the fluid diet from the other members of the colony, he found, as a rule, no protozoa. In the older larvae, soldiers, workers, and sometimes in the winged sexual forms,* protozoa are abundant and a wood diet is the rule.

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* It is interesting here to note the statement of Snyder (1920), regarding one of his cross-breeding experiments. He says, "A large series of young second form female adults of Reticulitermes flavipes, which possibly may have been fertilized by second form males, were taken from a fairly small colony and placed with mature first form dealated males, which had not copulated, in small shallow cells in decayed wood sunken in moist sand in glass jars and tin boxes.

After a period of ten days to two weeks all these second form females had died, but the first form males were still living and were active; they evidently were prepared to forage for themselves, whereas the second form adults needed the care and nourishment usually afforded them by the workers. Possibly the jaw muscles in these young second form adults had begun to degenerate through disuse in masticating wood. It is believed that second form males - like the females - would have succumbed, without the workers." First form adults, so far as my experience goes, harbor protozoa, but the second forms do not. This probably explains the inability of the second form adults to live without workers, that is, in the event the jaw muscles permitted them to eat wood. Of course the protozoa are lost, possibly because the host does not eat wood. I have experiments started which I hope will determine this point.

The wood, according to Imms, after being crushed by the mandibles and later by the gizzard, passes rapidly to the hind-gut without undergoing any chemical change. The pyloric valve prevents the readmission of wood particles into the mid-gut. To quote Imms: "On reaching the hind-intestine the wood is in a condition of minute fragments and particles, and the greater bulk of it gradually becomes taken up and absorbed by the numerous intestinal protozoa. Within the protoplasm of these organisms the woody material undergoes chemical changes, and, when ejected from the bodies of the protozoa, much of it is in a condition capable of being assimilated as food by the host termite. A significant fact is that lignous particles are not subjected to the action of the secretions of the mid-gut."

Imms further points out that, in his opinion, the experiments of Grassi and Foa (1911), where these authors claim to have kept Kalotermes (species not given, nor is the number of termites) alive and active for several months with all but a few of the intestinal protozoa killed off by incubation at about 35 degrees C., has no value, for during incubation the termites very probably subsisted on the large amount of fecal matter* which had accumulated in the jars where they

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* The fecal matter contains sugars and possibly other foods.

had been kept previous to incubation.

Imms, then, through an ingenious method of reasoning, reaches the conclusion that the protozoa are symbionts, a conception already reached by Buscalioni and Comes (1910), who claim, by means of various microchemical tests, to have shown that the wood is digested inside the bodies of the protozoa; and since the protozoa digest the wood, these authors conclude, that they are symbionts.* They state that Trichonympha agilis, harbored by Reticulitermes lucifugus, when treated with iodine dissolved in iodide of potassium, gives a characteristic glycogenic reaction in a region near the nucleus, and that this reacting region is sharply defined from the rest of the body. Cutler (1921) could not locate a definite glycogen reacting area in Pseudotrichonympha pristina, harbored by Archotermopsis wroughtoni, but, on the contrary, found that the glycogenic reaction was diffused through the entire organism. Grassi and Foa (1911) state that they in part attempted to verify the work of Buscalioni and Comes but that their results did not justify the drawing of any conclusions.

Dobell (1921) says, "When the association benefits both parties, the condition is one of symbiosis - a not very

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* A discussion of this may be found on page 30

frequent state in nature. An example is afforded by some of the flagellates living in termites. In return for the food and lodging which the termite gives to the flagellate, the latter helps the former digest its own food." Regarding this statement Dobell (1922)* says, "It is merely a general statement of my own personal view. It is not based on any particular experiments which I have made, but is an inference from what I myself have observed and others have recorded. For many reasons I consider it probable that the flagellates of termites aid their host in digesting the wood on which they feed. The observations of Buscalioni and Comes (1910) and of Imms (1919) especially seem to me to justify such a conclusion.

"However I am quite ready to admit that the matter has not been definitely settled beyond all question. In fact I recently discussed the problem with Dr. Koidzumi and we planned several experiments to determine the question... "

B. ORIGINAL OBSERVATIONS

At best some of the experiments of Buscalioni and Comes (1910) were conducted in a rather crude manner and, in

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* Personal communication.

addition to this, unwarranted conclusions were drawn due, often, to misinterpretation of the results obtained. The only conclusion which may be drawn from the results of their experiments is, that the protozoa digest the wood which they take into their bodies. But the fact that the protozoa digest the wood in no way hinders the termite from doing the same thing. Many animals ingest more food than they can use. The protozoa may feed from the wood particles in the termite's intestine, as one would naturally expect them to do, since nutrition with most of them appears to be holozoic. In order to establish the fact that the protozoa are symbionts, rather than commensals (feeding from the same table as their host), it must be shown that they actually aid their host in some way by doing something for the host which the host itself cannot do. The experiments of Buscalioni and Comes do not in any way fulfill this condition. They have only shown that the protozoa digest wood particles. The ability or inability of the termites to do the same thing was not studied. That the termites aid the protozoa, by furnishing them food and lodging, is obviously demonstrated by the fact that the protozoa, so far as known, cannot live outside of termites. But we cannot call the protozoa symbionts, until their ability to digest wood has been shown to be a necessary aid to the termite.

Realizing the difficulty of determining the relation of the protozoa to the termites so long as the two were associated together, several experiments were begun, with the hope of effecting a method for removing the protozoa from their host, and at the same time leave the host uninjured. It is needless to say that hundreds of experiments - some of which are given in table I - met with failure, for the host was either killed or severely injured in an effort to remove the protozoa from it.

When wood, previously soaked in a 5% aqueous solution of either sodium, potassium or calcium chloride, was fed to a colony of Reticulitermes flavipes (elsewhere in this paper - unless it is otherwise evident - when the words "termite," "termites" and "host" are used they refer particularly to this termite) harboring protozoa, within four to five days an occasional termite, free, or almost free, of protozoa was found, when many individuals from the colony were examined. The number free of protozoa increased as time progressed but, before all the termites in the colony had lost their protozoa, it was quite evident that the termites themselves were not normal. They had in some way been affected; whether by a direct action of the chemicals or by a gradual loss of the protozoa, it is

impossible to say from the data available.

About this time a much more promising method for removing the protozoa was evolved; namely, the incubation method, and the experiments employing the use of salts, etc. (see table I) to remove the protozoa, were discontinued. It might be well to mention here that, because of the success of the incubation method, many of the other attempts to free termites of their intestinal protozoa were, perhaps, not given a fair trial. If different dilutions of some of the chemicals had been used, the results, possibly, would have been more promising. Had x-ray and ultra violet light been used more extensively, excellent results might have been obtained.

It was found that all - not one was left alive - of the protozoa could be removed from the intestine of Reticulitermes flavipes by incubating them for 24 hours at 36 degrees C. This was a much more rapid method than the feeding of salts, as described above, where several days are required to remove the protozoa from all the individuals in a colony and, besides, the termites after incubation seemed to be perfectly normal in every way. All other attempts to remove the protozoa were now abandoned in favor of the incubation method, which may be briefly described as follows: Sometimes

stumps and large pieces of wood, containing thousands of termites, were brought to the laboratory without molesting or disturbing the colony. The stumps and large pieces of wood were placed in glass jars and incubated for 24 hours at 36 degrees C. At the end of the incubation period the jars were taken from the incubators, and, after many termites from each jar had been carefully examined to be certain that the protozoa had all been killed, were left at room temperature, which was usually about 20° C. Sometimes the stumps and logs were split before they were brought to the laboratory and the termites were taken from them and placed, with a good quantity of wood, in glass jars with metal tops. These jars were placed in the incubator as soon as they reached the laboratory. In other experiments, where termites were counted, small shell vials with cork stoppers were used, and from 10-100 termites were placed in each vial, and incubated in the same way that those in the large jars were. Several vials of termites without wood, and several with pure cellulose * (Whatman's filter paper) only, were incubated.

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* This paper was made by W.&R. Balston, Ltd., and contains .0004 grams of ash per circle of 55 mm. diameter. When "pure cellulose" occurs in this paper it refers to this grade of the genuine Whatman filter paper.

Other experiments in incubation were carried out with results as shown in table I. One very significant fact brought out by these experiments is that the thermal death point of the protozoa is 3-4 degrees C. lower than that of the insect host.*

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* Termopsis sp. from Ashland, Oregon, is killed at a lower temperature. It is killed in less than 24 hours at a temperature of 35.5 degrees C. Work on the T.D.P. of termites from various parts of the world is now in progress and the results will be published in a later paper.

Table I.

Methods and results of various attempts to free termites of their intestinal protozoa.

Method.	Number of experiments carried out.	Approximate number of termites used in each experiment.	Results.
Wood*5% NaCl	30	500	Kills protozoa in 10-15 days, but host is almost dead by this time.
Wood*5% $CaCl_2$	10	100	Same result as with NaCl.
Wood*5% KCl	20	500	Same result as with NaCl.
Wood*$HgCl_2$	20	500	Host killed in Dil. of 1/15,000.
Wood*$CuSO_4$	5	100	Killed host.
Quinine sulphate	2	100	Killed host.
Cinchona bark	2	100	Killed host.
Gentian violet	15	100	Dil. 1/100,000, host & protozoa unaffected.
Acid fuchsin	10	100	Dil. 1/100,000, host & protozoa unaffected.
Anhydrous $CaCl_2$ #	6	500	Many protozoa killed, host injured.
Conc. H_2SO_4 #	5	200	Many protozoa killed, host injured.
Hornet's nest	5	2,000	Host and protozoa unaffected in 2 mos.
Humus	10	10,000	Host and protozoa unaffected in 3 mos.
Soil	5	4,000	Host and protozoa unaffected in 2 mos.
Soap	3	100	Host killed.
Leather	3	50	Host & protozoa unaffected in 3 weeks.
Low temperature	5	200	2-3° C., no effect in 3 weeks.
Starvation	20	1,000	Many protozoa killed, host injured.
Ether fumes	4	100	1 min. no effect on protozoa or host.
Fumes of C_2H_5OH	4	200	2 min. no effect on protozoa or host.
Fumes of $CHCl_3$	2	50	$\frac{1}{2}$ min. no effect on protozoa or host.
U.V.Light, 10 in.	10	500	15 min. no effect on protozoa or host.
X-ray, 2 skin dos.	10	500	No effect on protozoa or host.
Inc@35°,5^{c}, 24 hrs.	15	5,000	Few protozoa killed, host uninjured.
Inc@36° C. 24 hrs.	-/*	100,000	All protozoa killed, host uninjured.
Inc@42° C. 10 min.	10	500	Few protozoa killed, host uninjured.
Inc@43° C. 10 min.	10	500	All protozoa killed, host uninjured.
Inc@44° C. 10 min.	10	500	Host not killed.
Inc@45° C. 10 min.	10	500	Host not killed.
Inc@46° C. 10 min.	10	500	Host not killed.
Inc@47° C. 10 min.	10	500	Host nearly all killed.
Inc@48° C. 10 min.	10	500	Host all killed, 48° C. is T.D.P. of host.

* Wood moistened with 5% Aq. solution of NaCl, KCl, $CaCl_2$ and with many dilutions of $CuSO_4$ and Hg Cl2.

# These chemicals were used in dehydration experiments.

-/* More than 2,000 experiments were carried out.

@ Incubated.

After incubation the termites were closely observed to note if they at any time became abnormal; to prevent the growth of moulds in the jars, for moulds are very destructive to termites; to be certain that the jars contained neither too much nor too little moisture - a thing that can only be learned from long experience in keeping large numbers of termites in captivity and an intimate knowledge of their habits in nature. Every known precaution was used to keep the termites during incubation in exactly the same environment, except temperature, as the unincubated controls. After incubation the incubated and the unincubated termites were kept under identical conditions, as regards food, temperature, moisture, light, etc.

The termites become abnormal in from five to fifteen days after they have been removed from the incubator. They are less active and their abdomens, if carefully examined, may be seen to be smaller and slightly flattened. The length of time required for abnormalities to appear depends on the kind of wood fed after incubation, the more decayed the wood the longer it is before any abnormalities appear. In two to five days after this first symptom is noticed the abdomen becomes still more flattened. Soon it is very much smaller and

almost flat, and two or three days later the animal can scarcely be made to move at all, for only a modicum of vitality remains. Death occurs, on the average, from 10-20 days after incubation. It occurs, as shown in table II, in some instances in less than ten days and in a few cases after twenty days, the longest being 27 days. In the less decayed wood, as mentioned above, death occurred early, and in the most decayed, it occurred late. But why did it occur at all? Why did the incubated termites die?

The death of the incubated termites - sterile, uninfected, uninfested and defaunated, so far as protozoa are concerned - may be due to the change in temperature, i.e., the incubation *per se*; it may be due to the killing off of all of the intestinal protozoa; or it may be due to the killing of intestinal bacteria and fungi, which, possibly, in some way aid their host in the digestion of wood and pure cellulose.

Kalotermes schwarzi, *K. jouteli* and *Prorhinotermes simplex* when incubated for 24 hours at 36° C. are defaunated, but enough material was not available to carry out extensive experiments on these forms as was done with *Reticulitermes flavipes*.

To be certain there was no error in the experiments which had been done up to this time, more than five hundred jars of termites were collected; some were incubated and some were not incubated, as shown in table II. No doubt 100,000 termites were used in these experiments and, in every case, death occurred, as in the preliminary experiments, within 10-20 days on the average after incubation. In no case did the unincubated and faunated controls die. In fact, they are still alive now, more than six months since many of the experiments were completed.

How may the cause of the incubated and defaunated (with the protozoan fauna removed) termites' death be determined? Is death a direct result of the incubation? That is, if the termites did not harbor intestinal protozoa which are killed off by the incubation, would death result in 10-20 days? In other words, is the death of the protozoa harbored by the termite in any way connected with or responsible for the death of their host; or is it an entirely independent phenomenon having nothing at all to do with the termite's death. In an effort to answer these questions many experiments were carried out, but only those experiments whose results throw some light on the questions involved will be mentioned and discussed.

Table II.

Showing the length of life of incubated and non-incubated termites when fed their normal diet of wood. 30 experiments selected at random from a series of 500.

No. of experiment.	Treatment.	Approximate No. of termites used in each experiment.	Length of life in days.	Remarks.
1a7	incubated	4,000	14	
2a7	unincubated	2,000	60	Jar became too dry due to neglect.
11a15	incubated	500	17	
7a15	unincubated	1,200	in*	Alive now, 9 months later.
12a15	incubated	400	15	
8a15	unincubated	1,000	in*	Alive now, 9 months later.
13a17	incubated	300	20	
14a17	incubated	500	20	
15a17	incubated	400	21	
1a17	unincubated	700	in*	Used in other Expts. after 6 months.
2a17	unincubated	2,000	in*	Alive now, 8 months later.
2a27	incubated	300	18	
2b27	incubated	200	21	
3b27	unincubated	700	in*	Alive now, 5 months later.
3a27	unincubated	500	in*	Alive now, 5 months later.
1d27	incubated	400	7	Mould caused early death.
2c27	incubated	600	15	
1a28	incubated	200	10	
2a28	incubated	100	14	
3a28	unincubated	200	45	Used in other experiments.
1a31	unincubated	1,500	in*	Alive now, 4 months later.
2b21	incubated	500	12	
3b31	incubated	700	18	
2a31	incubated	1,000	8	Wood decayed very little.
4a31	incubated	2,000	10	Wood decayed very little.
1c32	unincubated	2,000	in*	Used in other Expts. after 3 months.
2c32	incubated	1,500	27	Wood very much decayed.
1d32	unincubated	3,000	in*	Used in other Expts. after 3 months.
2d32	incubated	2,000	20	
1f33	unincubated	1,000	in*	Alive now, 4 months later.

* indefinitely.

Insects, like the cockroach, rather closely related to the termites morphologically, and many wood boring beetles, rather closely related to termites in habits, were incubated at the same temperature for the same length of time, and for a longer time, as were the termites. These were carefully observed for two months after incubation and no abnormalities were ever observed in any of them. It seems, then, that this temperature is not detrimental to these insects. But the fact that a cockroach or a wood boring beetle is not killed by the incubation method employed in killing the protozoa of termites, does not show that the termites themselves were not killed directly by incubation, because these insects do not harbor a vast multitude of intestinal protozoa that are suddenly killed off, as do the termites; and besides, they are, after all, quite different in some respects from termites.

Nasutitermes morio latreille, from Porto Rico, one of the Termitidae, and, of course, a termite which does not harbor intestinal protozoa, was procured for experimental purposes, the idea being that if this termite could withstand the same incubation temperature that Reticulitermes flavipes had been subjected to, and receive no ill effects therefrom, as evidenced by its ability to live indefinitely after incubation, the probability that Reticulitermes flavipes was not killed

directly by incubation was very much augmented. Then, some cause other than the effect of the increased temperature on the cells of the termite might be expected to be responsible for the termite's death. But Nasutitermes morio could not be kept in closed jars for a longer period than 5-10 days before death occurred, regardless of whether they had been incubated or not. Amitermes sp. from Uvalde, Texas, another genus of the Termitidae, was obtained, but it also could not be kept in closed jars. Other genera of the Termitidae, termites not harboring protozoa in nature so far as known, which might possibly be kept in closed jars - though nothing at all is known regarding this - were not obtainable. It then became necessary to answer in another way the question, does the heating kill the termite directly?

An effort was made to keep the incubated and defaunated termites alive by feeding them substances other than wood, their normal diet. Dextrose, peptone and starch, separately and altogether, were fed them; and it was evident, from the results (see table III) obtained by the feeding of these substances, particularly dextrose, that the lives of the incubated and defaunated termites had been prolonged.

But before these experiments had progressed very far a much better method of prolonging the lives of the defaunated termites was discovered, and no more experiments employing dex-

trose, starch and peptone were carried out. They were placed in glass jars and were fed a diet of fresh humus, which seemed to be a very satisfactory food, as the results tabulated in table III show. When they were given humus before any of the characteristic symptoms of wood fed defaunated termites had appeared, no such symptoms ever appeared. If these symptoms had appeared before humus was fed, the termites after being fed humus for three or four days became much more active, and within six to seven days they were normal again. Even when all the individuals in a jar of defaunated termites were almost dead, due to being fed on wood, many of the almost lifeless ones could be brought back to normal by feeding them humus. The humus fed defaunated termites appeared to be perfectly normal in every way long after all of their wood fed defaunated controls were dead. One hundred jars of defaunated termites were used in the humus feeding experiments, and from ten to more than a thousand termites were placed in each jar. It does not seem feasible or necessary to state when each of the one hundred jars were collected and incubated and just what happened to each of them, since the results of each experiment are similar, as table III shows.

Table III.

Showing the results of attempts to prolong the lives of incubated and defaunated termites by feeding them various substances. 27 experiments selected at random from a series of 100.

No. of experiment.	Substance.	Approximate no. of termites used in each experiment.	Length of life in days.	Remarks.
2a15	dextrose	200	30	
3a15	dextrose	200	34	
4a15	dextrose	200	40	
5a15	peptone	200	4	
10a15	dextrine	100	30	
7a15	dex&pep*	100	45	
9a15	dex&pep*	100	43	
3a19	dextrose	150	30	
4a19	dextrose	200	34	
4f33	humus	1,000	125	Experiment discontinued¢
4g33	humus	1,000	45	Allowed to become too dry
2g33	humus	500	125	Experiment discontinued¢
12a35	humus	1,000	60	Experiment discontinued¢
13a35	humus	1,000	60	Experiment discontinued¢
22a35	humus	1,000	60	Experiment discontinued¢
23a35	humus	1,000	60	Experiment discontinued¢
36a35	humus	1,000	40	Changed to a wood diet%
20a35	humus	1,000	45	Changed to a wood diet%
19a35	humus	1,000	20	Changed to a wood diet%
9a63	soil	1,000	50	Experiment not done with care
10a61	soil	200	45	Experiment not done with care
17a35a	int.ct**	50	40	Experiment discontinued¢
17a35b	int.ct**	50	45	Experiment discontinued¢
17a35c	int.ct**	50	34	Experiment discontinued¢
1a45	F.D.C.@	50	60	Experiment discontinued¢
2a45	F.D.C.@	50	60	Experiment discontinued¢
4a45	F.D.C.@	50	60	Experiment discontinued¢

* dextrose and peptone.

** intestinal contents of wood boring insect larvae.

¢ no evident signs of death at this time.

% death resulted in 10-20 days later.

@ fungus digested cellulose.

After the incubated and defaunated termites had been fed humus and kept alive for a longer period of time than they would have lived had they been fed wood, as shown by the death of all the wood fed defaunated controls; from time to time transfers back to a wood diet were made, with the result, in each case, of death in ten to twenty days, the usual length of life of wood fed defaunated termites. The last of the transfers back to wood was made after the defaunated termites had been kept alive, active, and apparently normal in every respect, on a humus diet for three months. Death resulted, as was expected from the previous experiments, and two weeks after all the defaunated termites which had been transferred from the humus to the wood diet were dead, the humus fed defaunated termites were alive and active, at which time the experiments were discontinued. That progressive development took place is evidenced by the fact that molting occurred in the humus fed defaunated termites three months after all the protozoa had been removed by incubation. Deeply pigmented first form reproductive adults were formed in several of the jars during December and January.

From these experiments it is obvious that the humus which was fed to the defaunated termites is potent to prolong their life four months - and probably indefinitely - beyond

the death point of such termites when fed a diet of wood. The unincubated controls, which had been fed wood and kept in the laboratory in the same way as the incubated and defaunated termites being fed wood and the incubated and defaunated termites being fed humus, were alive and active at the time the experiments were discontinued. Now, since the incubated and defaunated termites live indefinitely when fed humus, but die in two to three weeks when fed wood, and the unincubated and faunated termites live indefinitely when fed wood, it is evident that the death of the incubated and defaunated termites is not due directly to the incubation temperature, but to an acquired inability to make use of wood as food. In other words, the termites after incubation and the removal of the protozoa, are no longer able to maintain themselves on their normal diet of wood, and death results. The incubation has in some way incapacitated them as wood users. They feed on the wood, as may easily be determined by watching them or by examining their intestinal contents, but evidently are unable, for some reason, to utilize it - perhaps digest it, since humus, which is decomposed or digested wood, will keep them alive indefinitely.

When the protozoa are treated with a hydrochloric acid and phloroglucinol solution a distinct pink color appears

inside the bodies of the wood ingesting protozoa. Imms (1919) used this reaction to prove that the particles which he saw inside the bodies of the protozoa were wood particles - "ligneous particles." Oshima (1919) placed phloroglucinol and hydrochloric acid on the termite intestinal contents and interpreted the macroscopic pink color reaction, which appeared immediately after the solution was placed on the intestinal contents, as a positive test for lignin and, from this experiment, he drew the conclusion that lignin passes through the termite's (Coptotermes formosanus) alimentary canal unacted upon. Buscalioni and Comes (1910) interpreted the phloroglucinol reaction as indicative of the presence of wood sugars in the bodies of the protozoa, which sugars, these investigators claimed, the protozoa had by enzymatic action elaborated from the ingested lignocellulose particles. According to Sherrard (1922) no wood sugars can be obtained from wood except by extracting the cellulose. Mannan, for instance, very probably exists in the form of manno-cellulose. In every case the quantity of cellulose removed corresponds to the quantity of sugar produced. In other words, the amount of sugar obtained is proportional to the cellulose extracted. Ritter and Fleck (1922), in making an analysis of some American woods, obtained a yield (a mean of four samples)

of 6.82% of pentosan content from western yellow pine (Pinus ponderosa) extracted cellulose and a yield of 22.82% from tanbark oak (Quercus densiflora) extracted cellulose.

Aldopentoses, such as l-xylose and l-arabinose, when warmed with phloroglucinol and hydrochloric acid, give a cherry-red color to the solution. But this color reaction does not take place in the cold. Crocker (1921) states: "No case is known where materials conceivably present in wood, other than aldehydes, can react with the phloroglucinol reagent, in the cold, to produce a red color." Crocker reaches this conclusion after having tested a large number of chemically pure compounds. Klason* thinks the principal color-forming aldehyde of wood is coniferyl aldehyde. Cross and Dorée (1922) conclude: "We have therefrom a direct confirmation of the view that the color reactions of lignocellulose are those of derivatives and associated furfural products."

When tested with aniline acetate most of the wood ingesting protozoa are colored yellow, which coloration is probably brought about by the presence of methylfurfuraldehyde (Cunningham and Dorée 1914). When tested with the same chemical, at the same time, a small percentage of the wood ingesting protozoa and many of the wood particles free in the termite's (Termopsis) intestine are colored pink, which coloration is

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*Svensk Pappers-Tid; 23 (1920), p. 70.

probably brought about by the presence of a trace of furfuraldehyde, for Cross and Dorée (1922) state that "the aniline acetate test differentiates the aldehydes." Wood, before being ingested by the termites, gives the same pink color reaction to phloroglucinol and hydrochloric acid that appears inside the bodies of the protozoa. Also, aniline acetate reacts with uningested wood in the same way that it does with the wood particles inside the bodies of the wood ingesting protozoa and the wood particles free in the intestine.

Now, what does the phloroglucinol reaction show ? What is its value? It may show that the reacting substance (the substance producing the coloration) is a derivative of lignocellulose or a substance associated with lignocellulose. If the reacting substance is a derivative of lignocellulose, the lignocellulose bond or linkage has perhaps been broken, and it is highly probable that the protozoa are responsible for the breaking of it. Not many of the wood particles free in the intestine exhibit this reaction, and it is possible, of course, that the particles which do react have been inside the bodies of the protozoa. If the reacting substance is an aldopentose, as Buscalioni and Comes (1910) thought it was, it may be that the linkage or bond between aldopentoses and cellulose (Sherrard 1922) has been broken and, if so, it is

highly probable that the protozoa have broken it. But, since the wood, before being ingested by the termites and reingested by the protozoa, gives the same color reaction that it does after ingestion, it is perhaps best to suspend judgment until more is known regarding the specificity of the phloroglucinol reaction.

However, the clear-cut, definite, and unmistakable glycogenic reaction which appears inside the bodies of the wood-ingesting protozoa when treated with iodine in a solution of potassium iodide, is, perhaps, sufficient evidence to show that the protozoa exert a digestive action on the wood particles which they take into their bodies, for after the host has been fed cellulose (Whatman's filter paper. *Vide infra*) for three months glycogen is still present in the bodies of the protozoa and it cannot be demonstrated now, or at any time, in any of the cells of the host. The cellulose is probably split into cellobiose, the cellobiose into glycose, from which the glycogen is formed. The fact that no glycogen is present in the intestinal protozoa which do not ingest the wood particles or the cellulose particles, nor in the cells of the host, indicates strongly that the glycogen is formed inside the bodies of the protozoa that do ingest the wood particles or cellulose particles and that these protozoa

are responsible for its formation. The intestine of the host is slightly alkaline (pH 7.2) which would make acid hydrolysis impossible. The cellulose, then, must be acted upon by enzymes.

It has not been possible to demonstrate the presence of fats in the bodies of the protozoa, though the presence of fats in the intestinal and other cells of the host may be easily demonstrated. Those termites which receive a fluid diet from other members of the colony accumulate an abundance of fat.

It is highly possible that the protozoa are dependent on their host for proteins, since they live much longer on a cellulose-inorganic culture medium to which proteins have been added.

In studying many of the termites of Japan, Oshima (1919) came to the conclusion that a preference was exercised in favor of those woods containing a high percentage of cellulose. He is of the opinion that cellulose is the only constituent of wood which termites use as food. This opinion is based on the following experiment which he carried out. Camphor wood was analyzed and fed to Coptotermes formosanus; the nest which was constructed after the wood had been eaten was analyzed, and it was noted that the chief difference between the camphor wood and the nest was in the cellulose-lignin ratio. The wood

contained 48% of cellulose and 20% of lignin ($C_{40}H_{44}O_{15}$ Beckmann, 1921) while the nest contained 15% cellulose and 57% lignin. The method by which these percentages were obtained is not given, and for this reason we have no way of judging their accuracy. Regarding this experiment Oshima remarks: "It is quite obvious that the amount of cellulose is the main difference between the constituents of camphor wood and those of the nest. As there occurs no decrease of noncellulose, it is clear that cellulose has been taken as the food when camphor wood passes through the alimentary canal; and noncellulose, that is lignin, which is produced as a decomposed material of lignocellulose by special function of the alimentary canal, is discharged as the building material of the nest."

Obviously, Oshima's data do not warrant the conclusions which he draws from them, for it is impossible, without doing a quantitative experiment, to establish the fact that lignin is not used as food and that cellulose is, since both substances appear in the wood and in the nest. Oshima completely disregards - purposely or ignorantly it is impossible to say - all substances which are present in the wood except cellulose and lignin. The fact that lignin is present in the feces (and the test used is not a test for lignin at all) does not prove that it is not used by the termite as food, for cellulose is also

present in the fecal material and has to pass through the alimentary canal many times before it is used up. Perhaps it is all never converted into cellobiose and glucose. Then, it has not been shown that termites do not use lignin as food; nor has it been shown that they do.

Oshima (1919) fed cotton wool to *Coptotermes formosanus* and claimed that these termites lived more actively on this substance than when fed softwoods, the reason being, so he thought, that the softwoods have a lower percentage of cellulose. But the softwoods do not have a lower percentage of cellulose - at least those that have been analyzed do not have. However, hardwoods average about 100% higher in pentosan content than softwoods. But Oshima's experiment has no value whatever, since the number of termites used and the length of life, when given a cellulose diet (Oshima's diet of cotton[*]), is not stated. Many observers have noted

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[*] Purified cotton yields an ash ranging from 0.10 to 0.50 per cent, while raw cotton contains about 1 per cent of mineral matter. Raw cotton contains about 90 per cent cellulose and absorbent cotton 99 per cent. There is about 1 per cent of pentosan in purified cotton. Most any filter paper, then, is a better cellulose diet than cotton in any form. Some filter paper contains an extremely small percentage of ash. Of course it cannot be said that the cellulose of filter paper is "normal" cellulose. Cotton is really the only form of normal cellulose we have. Mayood and Cable (1922) have made a careful study of the cellulose of cotton and that of wood and conclude that the two are very similar, if not identical. The two, according to these investigators, are as much alike as the celluloses obtained from the same wood by acid cooking and by alkaline cooking. Until more is known regarding the nature of cellulose in various substances we may continue the usage of calling cotton "normal" cellulose.

that termites attack books, pasteboard, cloth, and wood pulp, but their ability to maintain themselves on these substances has not been investigated. Thinking it possible that termites in large numbers, when fed cotton,* do not maintain themselves solely on the substances present in the cotton, but continue to pass the wood, which they have previously eaten before being changed to a diet of cotton, through their bodies, digesting a portion of it during each passage, and thus giving the false impression of living on either the cotton or the filter paper, when in reality they were living on the wood particles which might still be present in their feces, I carried out the following experiment: One hundred termites were placed in 100 vials, i.e., one termite to each vial, and nothing but pure cellulose (Whatman's filter paper) was fed them. Many of these termites led an active existence and continued to harbor protozoa until the experiments were discontinued at the end of four months. Just how long termites can maintain themselves on a cellulose diet is not known, but is being investigated by the writer at the present time.

Another experiment, which was first done by accident, seems to throw considerable light on the direct cause of the death of the incubated and defaunated termites. Many of the

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* See footnote on page 52.

defaunated termites were being fed cellulose and a strong cellulose-decomposing fungus accidently got into some of the vials, and it was noticed that the termites in the vials containing the cellulose-digesting fungus did not die within two to three weeks. Some of them lived in the vials with the fungus for more than three months, but in the vials containing some of the same defaunated termites and cellulose which was not digested by a fungus, death occurred within two to three weeks. When this accidental and preliminary experiment was repeated (see table III) the same results were obtained. It seems quite evident, then, that incubated and defaunated termites cannot digest cellulose.

Now, since the incubated and defaunated termites remain alive and active indefinitely (see table III) when fed humus - which is wood (chiefly cellulose) digested or decomposed by bacteria, fungi, actinomyctes - and digested cellulose, but die when fed wood or when fed cellulose, it seems very probable that death is due to an inability to digest wood. Furthermore, it has been shown* that no sugars

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* Mannose from White Spruce cellulose: Sherrard, E. C., Report before the Am. Chem. Soci., Abstracted in Sci. Oct. 13, 1922.

can be gotten from wood except by first extracting the cellulose, which probably means that the sugars exist in combination with the cellulose. Then, we may reasonably conclude that the inability on the part of the defaunated termites to digest wood is due, perhaps, to the disappearance of cellulase, brought about, in some way, by the incubation and removal of the protozoa.

If cellulase is no longer present in termites after incubation - and it does not seem to be - what is responsible for its disappearance? In order to answer this question the two factors involved in bringing about the disappearance of cellulase, incubation and the removal of the protozoa, must be separated. Reinfection of the incubated and defaunated termites with protozoa seemed to be the principal key with which this puzzling question might be unlocked, but unfortunately for several months it was impossible to replace the protozoa in the termites, once they had been thoroughly removed. Finally, a rapid and easy method of reinfection was developed. Since the reinfection experiments are the most important ones in the paper, perhaps, one of the seven which were carried out, should be given in detail just as done and recorded in my note book. The other six are very similar to the one recorded here.

Ten large jars of termites were collected and were numbered 1-10p45. Numbers 1-7p45 were incubated at 36 degrees C. from 3:30 P. M., 10/21/22 to 3:30 P. M., 10/22/22. Numbers 8, 9 and 10p45 were not incubated and were left at room temperature (about 20° C.). Jars 1-7p45 were carefully examined on 10/23/22 and the termites in them were found to harbor no protozoa. In making this examination twelve individuals (workers), taken at random from each jar, were carefully dissected and their entire intestinal content painstakingly examined under the microscope for protozoa. If no protozoa were found, the colony was labeled "dafaunated termites." Jar 6p45 was used as follows on 9/24/22, the day after incubation: By means of a sieve the termites were separated from all pieces of wood and clumps of excrement larger than themselves. Then they were placed in beakers, and a piece of moist filter paper was placed in each beaker so that one corner just touched the bottom. The termites would leave the small pieces of wood and excrement and crawl up the filter paper, and when a large number of them were on the filter paper they were shaken from it and transferred to several small beakers. By this method the termites were entirely freed from all particles of wood and excrement. Now

they were taken, one at a time, from the small beakers and placed in watch glasses. Here the right antenna was cut off close up to the head, and one by one, after the right antenna had been removed, they were placed in shell vials until ten individuals were in each vial. In this experiment 14 vials with 10 defaunated termites each were used. These were numbered 1-14p49. Some unincubated and faunated termites were taken from jar 9p45, collected at the same time that 6p45 was and taken from the same colony of termites in nature. These were freed from wood and excrement in the same way that the defaunated ones were. Then 10 unincubated and faunated termites were placed with 10 incubated and defaunated termites in vials 1-11p49; in vials 12, 13, and 14p49 fifty unincubated termites were placed. Workers only were used in all the experiments. Whatman's filter paper, moistened with distilled water, was put in each vial; the vials were tightly corked and left at room temperature in the dark. Results: 10/24/22 at 5 P. M. three termites from vial 8p49 were examined. Two contained no protozoa and one contained a few. 10/27/22 all of the individuals - those previously defaunated and those from which no protozoa had been removed by incubation - in 11p49 were examined. Two were dead, 12 harbored protozoa, and 6

harbored no protozoa. Three of the 12 that harbored protozoa had the right antenna cut off. This was a clear-cut case of refaunation (replacing of the protozoa in the intestine after they had been removed by incubation). The number of protozoa present in these refaunated or reinfected individuals was about half normal.

On 12/29/22 six vials were examined with results as follows:

Number 3p49 contained 16 termites, 6 with no right antenna.
" 4p49 " 9 " , 7 " " " "
" 5p49 " 16 " , 7 " " " "
" 6p49 " 11 " , 5 " " " "
" 7p49 " 16 " , 4 " " " "

In number 14p49 fifty unincubated and faunated termites had been placed with the ten incubated ones and only two individuals with the right antenna cut off were taken from this vial, though others were present and might have been taken. Nearly all of the fifty unincubated individuals were alive. Now the termites with the right antenna removed which were taken from vials 3, 4, 5, 6, 7, and 14p49 to be counted were placed in separate vials 1-6p88 with a label on each vial designating the source. Whatman's filter paper was given them for food. They were observed regularly. All are alive

now, 2/7/23,* five weeks from the time they were separated from the unincubated. Two of the individuals in 3p49 were examined and were heavily infected with protozoa.

After association for several weeks with termites harboring protozoa the defaunated termites regain their protozoa, and, at the same time, their ability to live on a diet of pure cellulose or wood. Now, the important question is, how did the termites regain their ability to make use of pure cellulose or wood as food? More specifically, why do they now possess cellulase - and, perhaps, cellobiase? When the protozoa are removed from a termite's intestine, the ability to make use of pure cellulose or wood is lost, but when they are replaced, this ability reappears. Do the cells of the termite possess cellulase and cellobiase? It seems that they do not. Then where may these enzymes be found? The fact that their presence can only be demonstrated when the protozoa are present in the intestine of the host seems to indicate that the protozoa possibly possess them. If the protozoa do possess these enzymes, and the host does not, the way in which they aid their host is plain.

It seems evident that the protozoa aid their host in

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* Many are alive now 4/10/23.

some way to live on cellulose and wood, for it has been shown (table III) that defaunated termites can live indefinitely on the digestion products of pure cellulose or wood (humus). It now becomes highly probable that the protozoa aid their host in the digestion of pure cellulose and wood, since the host is able to live indefinitely on either of these substances only when it harbors the protozoa. But how do the protozoa aid their host in the digestion of wood? Mechanically, or by furnishing enzymes, as suggested above, which the host itself does not possess.

It is possible that the protozoa mechanically aid their host since the intestine is completely gorged with them. The intestinal cells of the termite may secrete enzymes such as cellulase and cellobiase, only when stimulated by actual contact with vast numbers of intestinal protozoa. Pavlovsky and Zarin (1922) in a study of the ferments in the alimentary canal of the bee, Apis mellifera, found catalase in the stomach and large intestine in winter only. Three hours after the first flight in spring, catalase was present in a very small amount, and two days later, not a trace was left. These authors claim that the discharge of catalase in the rectum depends upon the accumulation of feces in it during hibernation. The bee has no need for this enzyme

except during hibernation, when it seems to regulate the different oxidizing processes and destroys the surplus peroxides as they accumulate. The production of any enzyme may be explained either as a "hunger phenomenon" or due to favorable conditions of nutrition, the latter being, perhaps, the better explanation. Young (1918), in his work on inulase, showed that stimulation to increased production of this enzyme was dependent upon a direct chemical stimulus - substances present in the medium. It has been impossible to extract cellulase or cellobiase from either those termites harboring protozoa or those not harboring protozoa (defaunated termites). Pringsheim (1912) claims that cellulase is an endoenzyme and is secreted from the cell when stimulated by direct contact with cellulose. This perhaps explains my extraction failures.

The other way the protozoa may aid their host is to digest the wood for it. Since it has been shown that defaunated termites cannot digest wood, the experiments of Buscalioni and Comes (1910), where these investigators demonstrated that the wood particles ingested by the protozoa are digested, are now of great value, though they were worthless so long as it was not known that the host could not digest wood without the protozoa. There are many reasons for thinking that the protozoa digest the wood for their host. In Reticulitermes flavipes practi-

cally all the wood particles which reach the hind-intestine are immediately ingested by the protozoa. Hundreds of individuals of this species have been examined to note if wood particles were ever present in a very great quantity in the intestine, and in only a few instances were they ever found in abundance. Perhaps, directly after the termite has taken a meal, wood particles may be quite plentiful in the intestine, but it is certainly not long before nearly all of these particles of wood are taken into the bodies of the protozoa. In Termopsis many wood particles are free in the intestine, though the wood ingesting protozoa harbored by this genus always have their bodies filled with wood particles to the same extent as do the wood ingesting protozoa harbored by Reticulitermes. Quite a bit of woody material is present in the expressed pellets of excrement of Termopsis, whereas Reticulitermes, on the contrary, has a liquid excrement.

Of course the mere fact that the protozoa take the particles of wood into their bodies does not mean that they in any way alter these substances, for many protozoa ingest substances which they cannot use as food. Not all of the termite protozoa ingest solid particles of wood for food, or at all, though most of them do. Among the protozoa harbored by Termopsis sp., of Ashland, Oregon, Streblomastix strix does

not ingest the wood particles, and this organism never gives a glycogen reaction. Streblomastix strix, then, may get its nourishment from the digestion products of the other protozoa which Termopsis harbors, such as Trichonympha campanula, which ingests great quantities of wood particles and always gives a very clear cut and definite glycogen reaction, provided its host has not been wood starved; or it may derive its nourishment directly from the products which its host has assimilated, in which case it is a true parasite. If this protozoon gets its nourishment from the digestion products of other protozoa present in the intestine of its host, just in the same way that the termite gets its nourishment, Streblomastix strix, then, may be a commensal from the viewpoint of the termite, but from the viewpoint of its relation to the wood digesting protozoa, it is a true parasite, since it in no way aids the wood digesting protozoa, whereas the termite does aid them since it furnishes the wood which they digest. Of course Streblomastix strix may use both the products assimilated by the termite and the products present in the termite's intestine as a result of the digestion of wood by other protozoa, which products have not been assimilated by the termite. Those individuals of Trichonympha campanula containing the greatest quantity of wood particles in their bodies give the most distinct glycogen reaction. The reaction is much more

pronounced in the posterior half of the body where most of the ingested wood remains. Another form, *Trichomonas termitis* (*Trichomonas* because this organism has an undulating membrane, axostyle and three flagella and is not the same organism as *Trichomitus termitidis* which Kofoid and Swezy (1919) described from *Termopsis angusticollis* of Berkeley, California), ingests wood particles but does not give the glycogen reaction except in a very few instances. As a matter of fact, about one individual in a hundred examined will give a slight glycogen reaction. This organism probably does not make use of the wood particles, but, perhaps, ingests them while feeding on bacteria, since it has a cytostome, an organelle which *Trichonympha*, *Dinenympha*, *Streblomastix*, *Pyrsonympha*, and most of the other genera of termite protozoa, do not possess.

Among the protozoa harbored by *Reticulitermes flavipes*, *Trichonympha agilis* and *Pyrsonympha vertens* ingest wood particles and give a glycogen reaction. Several of the species of *Dinenympha* do not ingest wood particles or give a glycogen reaction. When these termites (*Termopsis* and *Reticulitermes*) are wood starved, the protozoa (*Trichonympha campanula*, *T. agilis*, *Pyrsonympha*, etc.) that give the glycogen reaction always die off first. Sometimes practically all of these forms are dead before any dimunition in the other forms occurs. *Pyrsonympha* is probably of great value

to its host owing to its peculiar habit of attaching itself to the chitinous layer investing the inner surface of the intestine. Individuals of this genus, instead of passing out of the intestine with the feces when they die, remain attached to it long after death, and thus the food which they have prepared for their host is kept in the intestine until the host has an opportunity to use it. The attachment is, perhaps, also quite useful to the protozoon, since this organism is not provided with numerous flagella as is Trichonympha and by means of the attachment apparatus it is thus enabled to maintain its position in the intestine in the complex struggle for existence among the many species of protozoa which completely fill the lumen of the termite's intestine. No doubt Pyrsonympha, Trichonympha and others, die by the hundreds daily, and thus give over to their host many substances which they have obtained from the wood particles. That they give sugars,* such as xylose, may be shown by testing with phloroglucinol and hydrochloric acid. But it is not the intention of the writer to show in this paper the relation of each species of protozoa to its host. Not all of the protozoa harbored by the two hosts, Termopsis and Reticulitermes, have been mentioned, and the protozoa of other termite genera have not been mentioned at all. In a later paper this question will be considered in more detail.

* If the work of Buscalioni and Comes is accepted.

Unwilling to conclude definitely that the protozoa were entirely responsible for the digestion of cellulose, the other microorganisms harbored by Reticulitermes flavipes were studied. Bacteria were sometimes numerous, and these were studied in many ways. Incubation at 36° C. for 24 hours does not seem to affect their numbers. All the known methods, aerobic and anaerobic, for isolating cellulose decomposing bacteria were given more than fifty trials, but, since the results of all these experiments were negative, no tabulation has been made. An inorganic medium composed of

K_2HPO_4	1.00	gram
$MgSO_4$	0.50	"
KCl	0.50	"
$FeSO_4$	0.01	"
$NaNO_3$	2.00	"
H_2O	1000.00	cc.

to which cellulose was added in two forms: a small piece of Whatman's filter paper was placed in the test-tubes containing the inorganic medium, and a 0.5% cellulose suspension was added to the inorganic medium. This was sterilized in the usual way and the inoculations made. Then the cellulose suspension and inorganic medium plus agar sufficient to make a solid medium were used. When cellulose was the only carbohydrate present, no growth ever occurred; but when other carbohydrates, such as dextrose, were present a rapid growth took place. For more information regarding the methods used in

studying cellulose decomposing microorganisms the reader is referred to Waksman (1919 and 1922*), Kellerman (1913), McBeth (1913 and 1916), Brown (1915 and 1917), Schmitz (1919), and others. After employing all of these methods it was concluded that no cellulose digesting bacteria are harbored by *Reticulitermes flavipes*, since more than one hundred individual attempts to isolate cellulose decomposing bacteria met with failure, even after the cultures were more than two months old. It was an easy matter to isolate such bacteria from the soil and from cow chips.

An attempt was also made to isolate cellulose decomposing moulds and actinomyctes from termites, and this attempt was a failure. This experiment was repeated ten times with the same result each time.

None of the known cellulose digesting bacteria, moulds and actinomyctes die at 36 degrees C. As a matter of fact this is the optimum temperature for many of these organisms. In some instances the cellulose is decomposed much more rapidly at even a higher temperature.

These negative results indicate that *Reticulitermes flavipes* harbors no bacteria, moulds or actinomyctes capable of decomposing cellulose.

The most logical conclusion, then, from the results of all of the experiments carried out, is that the protozoa

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* This paper contains 957 references.

actually aid their host by digesting the wood - or more specifically by breaking up the cellulose molecule and freeing many substances which are bound to it in some way - for it, because the host itself cannot digest cellulose. The protozoa can digest cellulose.

The relationship, then, between *Reticulitermes flavipes* and some of its intestinal protozoa, particularly *Trichonympha* and *Pyrsonympha*, is one of symbiosis. The termites are able to maintain themselves on their normal diet of wood only when they harbor protozoa to digest the wood for them. The protozoa are harbored only when their host eats wood or the excreta of other individuals containing wood and protozoa. The larvae must become infested with protozoa, by eating the feces of older and infested members of the colony, before they can maintain themselves on a strict wood diet. The host procures the wood for the parasite, and the parasite digests it for itself and for its host. Each is a servant to the other; the protozoa are dependent on the termites for food and lodging, and the termites are dependent on the protozoa for the protozoal cellulose digestion products.

GENERAL DISCUSSION

The number of protozoa present in a single host is truly enormous. Here we have an animal whose intestine is completely filled with flagellate protozoa, the majority of which live in a symbiotic relation to their host. Perhaps many - if not all - intestinal flagellates - human as well as animal - are harmless to their host. At least their presence does not mean anything - harmful or otherwise. Only careful and painstaking investigation will reveal their relation to their host.

The examples of symbiosis in nature hitherto described, such as algae and fungi (forming lichens), hydra and zoochlorellae, sea-anemone and hermit crab, are well known. These relationships are much closer than such relationships as ants and aphids, insects and flowers, etc. The symbiosis exhibited by termites and their intestinal protozoa is as real or true as any yet described. How such a relationship was developed is an extremely interesting but very difficult question to consider - or even theorize * on - for

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* Since none of the protozoa of termites are known to occur elsewhere it is interesting to speculate on their origin. Where did these insects get them? The ability of soil protozoa to digest cellulose has not been studied and little is known regarding the protozoa of plants. If there

it has undoubtedly been established a very long time.

The ability of animals to live indefinitely with a sterile intestine is still a live, debatable question. Osborn and Mendel (1914 b) have shown that microorganisms are of value to higher animals as elaborators of protein from non-protein substances ingested. Armsby (1911) showed that non-protein substances are a source of protein to herbivorous animals, probably due to the formation of digestable bacterial protein in the digestive tract. There is certainly a very small amount of protein in wood, and

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are protozoa parasitic on plants, or protozoa in the soil, capable of digesting cellulose, it is possible that some of them became inhabitants of termites. It may be that the symbiosis was established in this way. These insects, before harboring protozoa, fed on soil and wood and, perhaps, were able to utilize some of the substances in the wood and many of those in the soil. The intestine of termites, feeding on practically the same food that the protozoa free in nature feed on, is certainly a much more constant - and hence better - environment for the protozoa than soil, or even plants. But, if the termites once possessed the ability to digest cellulose, why did they lose it? If the acquired parasites had been digesting cellulose longer than the termites and could do it better, i.e., more completely, this may be responsible for the loss of the ability to digest cellulose. The insects probably lost their ability to secrete enzymes, such as cellulase and cellobiase, because it became unnecessary for them to do it. However, it is also just as reasonable to suppose that the termites never possessed the ability to digest cellulose, since so few animals do.

Another possibility, and perhaps a more plausible one, is that the termites, before they became infected with protozoa, probably fed on humus. Some wood was ingested - though not digested - along with the humus. The protozoa

it may be that termite protozoa aid their host in some such fashion as intestinal bacteria aid their host. Kianigin (1917) reopened this question by a review of all the literature. The greatest quantity of microorganisms - in the case of mammals and other vertebrates - is located in the non-digestive portions of the alimentary canal. Also the increasing number of animals which may be raised aseptically indicates that an aseptic existence may be possible in the majority of cases. Bacteria carry decomposition to a lower level, yielding unassimilable substances such as methane, carbon dioxide, indol, skatol, phenol, kreseol, etc. Perhaps the chief beneficial role of the intestinal flora is the synthetic powers of the microorganisms.

But the relation of many insects to microorganisms is a different question, and at least from one viewpoint, if from no other, a more interesting one and also one which may be studied more satisfactorily. Baumberger (1919) has shown

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were taken in and found the environment suitable. Rapid multiplication and a few deaths in the more suitable environment, soon filled the termites with them. The wood feeding habit of the termites gradually increased. At present every conceivable step from non-wood feeding termites to strict wood feeders, is well illustrated in the various termites.

But what happened to the ancestors of pre-termite protozoa - the organisms which lived in the soil or in wood? They have probably evolved along another line, in an opposite direction from those individuals who became inhabitants of termites.

"that Drosophila living in fermenting fruit are dependent for their food supply on the synthetic and absorptive powers of yeast cells." In a similar manner his study of "the relation of Musca domestica to manure, of Desmometopa to decaying meat, and of Sciava and Tyroglyphus to decaying wood shows clearly that these arthropods also feed on microorganisms." However, microorganisms are not associated with these insects as symbionts - certainly not in the same sense or degree that termite protozoa are associated with their host - but as food.

The nitrogen content of birch-wood is very high as compared with that of other woods. It is 0.108%. In many woods it runs as low as 0.08%. From the work of Baumberger (1919) and others, it is evident that many insects, due to the low protein content of the substratum on which they live and feed, are dependent on microorganisms for the proteins which they contain. Since such a small amount of protein is present in the food of termites it is quite likely that they, too, are dependent on microorganisms for a part of their proteins. Fungi may often be seen in the intestine of many termites.

The relation of ambrosia beetles to fungi has been studied by Hedgcock (1906) and others, and the fungus growing habits of the Termitidae have been studied by many investigators,

chief of which are Doflein (1905 and 1906), Petch (1906) and Escherich (1909), but the exact relation of these insects to the fungi is not known. The fungi may simply furnish food (protein) for the insects, and, on the other hand, they may elaborate substances from the wood and grasses which the beetles and termites cannot elaborate, which substances are very important in the insects' metabolism.

Internal symbionts (yeasts) have been described from the beetle Anobium paniceum, by Karawaiew (1899) and Escherich (1900), and Portier (1905) claims that a micrococcus and a fungus live symbiotically with the caterpillar Neptiluca, but in no instance have intestinal flagellates been shown to live symbiotically with their host. The flagellates living in termites are the first example of such a relationship.

As a rule the second and third reproductive forms do not harbor intestinal protozoa. Why? For one of two reasons perhaps: (1) the liquid diet upon which these individuals are fed kills the protozoa; (2) the failure of the host to eat wood causes the death of the protozoa. There is more evidence in favor of the latter view. In the first form reproductive adults protozoa are usually present - though never in abundance as in workers and soldiers - and this form eats some, though never as much wood as the workers and soldiers. When workers

and soldiers are wood starved the wood ingesting protozoa which they harbor die first. When workers are fed cow chips the protozoa disappear in three to four weeks. But here, as in the case of saliva, it is impossible to determine whether the change in the intestinal content brought about by the change of food, results in the loss of protozoa, or whether the failure of the host to provide the protozoa with wood results in their death. Since there is a great deal of cellulose in the cow chips it is more likely that the change in the intestinal content is responsible for the death of the protozoa. The bacteria of the cow chips do not kill the protozoa, for sterile cow chips are just as effective in removing them. This question is now being attacked from many angles and it is hoped that a more definite answer may result.

There is considerable difference of opinion regarding the extent to which cellulose is utilized in the animal organism. Very little is really known about the mechanism which vertebrates - and most, if not all, invertebrates too - employ in making use of cellulose. It is interesting in this connection to compare the views expressed by two prominent physiological chemists. Hawk (1921) states that many of the herbivora use as much as 25% of the total ingested cellulose,

that less than 5% is used by dogs and the amount used by man is "too small for it to play a role of importance in the diet of a normal individual." He says: "In neither man nor the lower animals has there been demonstrated any formation of sugar or glycogen from cellulose." Von Furth (1916) states that 30-70% of the cellulose eaten by herbivorous domestic animals is digested, and that man digests about 50% of the cellulose which he consumes, and in cases of habitual constipation he may digest as much as 80%. The filtered mammalian intestinal content is inactive to cellulose (Pringsheim, 1919). Pringsheim (1919) also claims that no cellobiose-splitting enzymes are present in any of the organs of cattle. Biery (1914), Biery and Giaja (1912) and Billard (1914) report the presence of cellulose-splitting enzymes in hepatic secretions of certain mollusks and crustaceans. Harrington (1921) claims to have demonstrated the presence of cellulose-splitting enzymes in _Teredo_. Dore and Miller (1923) made a comparative analysis of the wood eaten and the borings passed by _Teredo navalis_, and, as a result of the analyses, these investigators conclude that "the wood lost about 80% of its cellulose, and from 15-56% of its hemicelluloses, including from 11 to 40% furfural yielding constituents such as pentosans, etc," during its

passage through the animals' digestive tracts. They state further that "the simplest explanation of the disappearance of this carbohydrate material is that the cellulose and hemicelluloses of wood are partly digested by the teredo and probably hydrolyzed to simple carbohydrates which the animals can use." In all these investigations it should be noted that many substances other than cellulose were present. In no instance was cellulose alone fed the animals. It would be very interesting, indeed, to study the microorganisms of these crustaceans and mollusks. No mention is made by these investigators regarding the possibility of microorganismal cellulose digestion.

Recently a cellulose digesting anaerobic bacterium has been isolated from 60% of the human stools examined (Khouvine-Delaunay, 1922), and it is quite possible that future researches will reveal that intestinal microorganisms perhaps play a more important part in cellulose digestion than has previously been thought. Intestinal bacteria and fungi quite often aid their vertebrate and invertebrate hosts in the digestion of cellulose, and since it has been shown in the present investigation that intestinal protozoa can digest cellulose, it is now possible that the Infusoria, such as _Diplodinium_, _Entodinium_, _Butschlia_, _Isotricha_, _Dasytricha_ and _Ophryoscolex_, harbored by ruminants, notably the ox, goat, sheep,

camel and reindeer, may aid their hosts in the digestion of cellulose and hemicellulose. The Equidae also harbor similar infusoria.

GENERAL SUMMARY

1. There are four families of termites and all the species and genera of three of them, Kalotermitidae, Rhinotermitidae and Mastotermitidae, that have been examined have been found to harbor enormous numbers of intestinal protozoa. No termite of the other family, Termitidae, has been found to harbor intestinal protozoa.

2. The principal food of protozoa harboring termites is wood and the principal compound in the wood which the termites use is cellulose. This was demonstrated by keeping termites alive and active indefinitely on a cellulose diet.

3. The protozoa harbored are all killed off by incubation at 36 degrees C. for 24 hours, while the termites apparently are not injured at all by the incubation.

4. The incubated and defaunated (with the protozoan fauna removed) termites die within 10-20 days, on the average, after incubation, if fed their normal diet of wood.

5. When the incubated and defaunated termites are fed digested wood (e.g., humus) or fungus digested cellulose, they

live indefinitely.

6. The death of the incubated and defaunated termites is not due to the incubation per se, but to an inability to digest wood.

7. When the incubated and defaunated termites are reinfected with protozoa their ability to utilize wood, their normal diet, reappears, and they live indefinitely.

8. The removal, then,of the protozoa seems to be responsible for the loss of the ability to utilize wood as food. To determine this question the ability of the bacteria, fungi and protozoa, harbored by termites, to digest wood or pure cellulose was carefully studied. It was found that the bacteria and fungi could not digest cellulose, but that some of the protozoa could.

9. Now, since the termites die in 10-20 days, if fed a wood diet, after the protozoa have been removed from them because they cannot digest their food (wood), as shown by the fact that they do not die, but live indefinitely, when fed digested wood or when reinfected with protozoa and fed wood, and since the protozoa do digest the wood particles which they take into their bodies, it is highly probable, if not certain, that the termites are dependent on the protozoa to digest their food for them.

10. Not all of the protozoa harbored by *Reticulitermes flavipes* digest wood particles. Some of them either live at the expense of the wood digesting protozoa or their host, or both.

11. The protozoa receive from the termites food and lodging, for which they give in return protozoal wood digestion products.

12. The relationship between some of the protozoa, particularly *Trichonympha* and *Pyrsonympha*, and their host, *Reticulitermes flavipes*, is one of symbiosis.

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BIOGRAPHY

It is customary at this school for the writer of a doctor's dissertation to include some information regarding his personal history. I was born at Union, Mississippi, November 14, 1892, and have attended the following schools: the University of Mississippi, where the B. S. degree was granted me in 1917, the University of Chicago and the Johns Hopkins University. My teaching experience. as Instructor in Zoology at Emory University and Kansas State Agricultural College. covers a period of three years.